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THE DEVELOPMENT AND APPLICATION OF A RADIOIMMUNOASSAY FOR 5α-ANDROST-16-EN-3α-OL IN PLASMA

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SUMMARY

A radioimmunoassay (RIA) for 5α-androst-16-en-3α-ol in human and porcine plasma has been developed. Antibodies were produced in rabbits immunized against 5α-androst-16-en-3-(O-carboxymethyl) oxime conjugated to BSA. The antiserum cross-reacted with other 16-androstenes as follows: 5α-androst-16-en-3α-ol, 42%; 4,16-androstadien-3-one, 23.8% 5α-androst-16-en-3β-ol, 16.1% and 5,16-androstadien-3β-ol, 1.19%.

Although the method gave an acceptable standard curve for 5α-androst-16-en-3-one (10–1000 pg), it was not possible to separate by t.l.c. an unknown contaminant in plasma which interfered with the RIA of the steroid. However, by exploiting the high (42%) cross-reaction with 5α-androst-16-en-3α-ol, a RIA has been developed involving a thin layer chromatographic step. Regression analysis of the data in an accuracy study gave the equation $y = 0.986x + 151.5$, with $r = 0.999$, while the coefficients of variation of replicate assays of plasma 5α-androst-16-en-3α-ol were in the range 9.5–15.6%.

The mean values for plasma 5α-androst-16-en-3α-ol in 31 healthy men and 16 healthy women were 3.08 (range 0.3–14.9) and 0.66 (range 0–2.4) ng/ml, respectively. In boars, sows and castrated male pigs, the corresponding mean values were 30.7 (range 10.9–58.9), 0.24 (range 0.08–0.77) and 0.27 (range 0.06–0.51) ng/ml, respectively.

INTRODUCTION

Two odoriferous 16-androstenes were first isolated from boar testes by Prelog and Ruzicka[1]. Since that time 5α-androst-16-en-3-one (5α-androstenone) and 5α-androst-16-en-3α-ol (an-α) have been shown to have an important pheromonal role in the mating behaviour of pigs [2, 3] and it has become necessary to estimate the concentrations of these steroids and of other 16-androstenes in the plasma and tissues of these animals.

Initially, an-α was estimated in the urine of men and women by a colorimetric method [4] and more recently by a gas–liquid chromatographic method [5]. This has now been extended by various workers for the determination of 5α-androstenone, an-α, 5α-androst-16-en-3β-ol (an-β), 5,16-androstadien-3β-ol (andien-β), or 4,16-androstadien-3-one (androstadienone) in testis of humans [6], rats [7] and pigs [8] and in adipose tissue of pigs [9]. In 1974, however, Andresen [10] described a radioimmunoassay (RIA) for 5α-androstenone and has measured the concentration of this steroid in peripheral blood plasma of boars, sows and castrated male pigs, and has recently applied the method to adipose tissue of boars [11]. The antiserum, however, cross-reacts to the extent of 100% with androstadienone, which may conceivably also be present in the tissue or plasma to be analysed. Furthermore, no attempts were made to separate the 16-androstenes during the assay procedure.

The present paper describes the development and use of a RIA which was initially intended to be specific for 5α-androstenone but which had to be modified subsequently to measure specifically an-α.

A preliminary report of this work has been published already [12].

EXPERIMENTAL

Materials. [5α-^{3}H]-5α-Androst-16-en-3-one (15.9 Ci/mmol) 90% purity) was generously supplied by Dr. W. Hafferl, Syntex Research, Palo Alto, Calif., U.S.A. Purification was achieved first by chromatography on alumina columns and secondly by t.l.c. on Kieselgel G (Merck, Darmstadt) in benzene–diethyl ether (9:1, v/v) run twice [13]. A single peak of radioactivity was then detected by radio-scanning which coincided with the position of authentic 5α-androstenone used as marker. [5α-^{3}H]-5α-Androst-16-en-3α-ol was prepared from the purified tritiated ketone by borohydride reduction [14].

Bovine serum albumin (BSA), in solution, was supplied by Armour Pharmaceutical Co. Ltd., Eastbourne, U.K. Dyes for markers in t.l.c. were obtained from British Drug Houses Ltd., Poole, Dorset, U.K. Freund's Complete Adjuvant was purchased from Difco Laboratories, Detroit, Michigan, U.S.A. Dextran T-70 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden and Norit A from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Some authentic 16-androstenes were synthesized in the laboratory by methods reviewed earlier [15] and others were obtained from Dr. C. L. Hewett, Organon Laboratories, Lanarkshire, Scotland.

Methods. The buffer used in the assay (see below) consisted of 50 mM Tris–HCl (pH 7.4) containing NaCl (0.9%, w/v), sodium azide (0.1%, w/v) and BSA (0.4%, w/v). The Dextran-coated charcoal suspension used for removing all unbound steroid was prepared from 50 mM Tris–HCl (pH 7.4) containing dextran T-70 (0.5%, w/v) and washed Norit A (2.4%, w/v). Radioactivity was measured with a Beckman Automatic Scintillation Spectrometer (Series 200), Using either sulphur-free xylene containing 2,5-diphenyloxazole (0.3%, w/v) for non-aqueous samples or toluene-Triton X-100 (2:1, v/v) containing 2,5-diphenyloxazole (0.2%, w/v) for aqueous samples.

Preparation of antigen. The method used earlier [16] for the synthesis of testosterone-3-(O-carboxymethyl)oxime was utilised in the preparation of 5α-androst-16-en-3-(O-carboxymethyl)oxime, as described by Andresen[10]. This derivative was then conjugated to BSA by the mixed anhydride technique [10].

Immunization. The 5α-androstenone-3-BSA conjugate (2 mg) was dissolved in normal saline (1 ml) and mixed with Freund's complete adjuvant (2 ml). The suspension was thoroughly mixed by repeatedly drawing it into and expelling it from a syringe and needle (size No. 1, Gillette, London, U.K.). A drop of the suspension was placed on water to test for stability and, if this test proved acceptable, the remainder of the suspension was injected into a rabbit in at least six sub-cutaneous sites. Booster injections were given every four weeks for a period of sixteen weeks. Fourteen days after each injection, a sample of blood was taken and the serum tested for binding activity.

Preparation of antiserum. Approximately 50 ml of blood was obtained from the rabbit two weeks after booster injections. After separation, the serum was precipitated with respect to BSA as described earlier [17] and serial dilutions (1:10 to 1:10000) were prepared with Tris-HCl buffer (see above). [5α-^{3}H]-5α-Androstenone (5000 c.p.m.) dissolved in ethanol (100 μl) was added to glass tubes and the solvent evaporated in a vacuum desiccator. The dilutions of antiserum were then added, vortex-mixed and incubated for 1 h at 37°C then overnight at 4°C. The unbound steroid was removed by adding Dextran-coated charcoal suspension (100 μl), vortex-mixing and standing for 15 min at 4°C followed by centrifuging for 10 min at 2000 *g* at 4°C. The supernatant was decanted into a counting vial, toluene—diphenyloxazole-Triton scintillant added and radio-activity measured. The displacement of labelled steroid was established by adding 5 ng of unlabelled 5α-androstenone to tubes at the same time as the tritiated steroid, then proceeding as described above.

Standard curve. Standard solutions of 5α-androstenone in ethanol were prepared containing 10 to 1000 pg per 100 μl. One hundred μl of each concentration was added to a series of glass assay tubes in duplicate, while zero tubes contained only ethanol (100 μl). [5α-^{3}H]-5α-Androstenone in ethanol (100 μl, 5000 c.p.m.) was then added to every tube and the solvent evaporated in a vacuum desiccator. A 1:4000 dilution (see Results) of the antiserum (0.5 ml) was added to each tube, and the incubation and removal of unbound steroid performed as described above. After counting, the bound steroid was expressed as c.p.m. plotted against log pg of unlabelled steroid (Fig. 1).

Chromatography. In view of the cross-reaction found with other 16-androstenes (Table 1), a chromatographic step was essential in the assay. For this purpose column chromatography on Sephadex LH 20 [ref. 18] was attempted, using benzene–ether (9:1, v/v) or light petroleum (b.p. 80–100°) as eluting solvents. However, no resolution of 16-androstenes was achieved. Chromatography on columns of Lipidex™ [19] and alumina [5] resulted in acceptable separations but high blanks (> 500 pg per tube) were obtained.

By means of t.l.c. in dichloromethane–ethyl acetate (9:1, v/v), separations of 16-androstenes were achieved, with the following R_f values (±S.D.): 5α-androstenone, 0.87 ± 0.02; androstadienone, 0.74 ± 0.04; an-α, 0.68 ± 0.02. The use of a marker substance made the location of an-α easier on the plate and, for this purpose, it was found that a contaminant of Sudan IV dye had an R_f value (0.67 ± 0.01) equal to that of an-α. The contaminant was therefore separated from the dye by t.l.c. in dichloromethane–ethyl acetate (9:1, v/v) and a solution (0.5 mg/ml) prepared in ethanol. Twenty μl of this was added to all samples and blanks prior to the chromatographic step. The dye was checked in the scintillation systems used for radioactive counting but no loss in counting efficiency due to quenching was found. The dye contaminant also had only minimal influence on the antiserum used since the cross-reaction was found to be less than 0.01%. Using the t.l.c. separation and the marker dye, the blanks have been reduced to 20–25 pg per tube.

Radioimmunoassay for An-α. In our initial attempts to produce a RIA for 5α-androstenone, it became clear that a substance in plasma was extracted together with 5α-androstenone and could not be separated from it by t.l.c. The results obtained were found to be much greater than the highest standard (1000 pg per tube). For this reason a RIA for 5α-androstenone was abandoned and an assay produced for an-α exploiting the high cross-reaction (42%) that this steroid showed with the antiserum.

[5α-^{3}H]-An-α (500 c.p.m.) in ethanol (100 μl) was placed in glass assay tubes and the solvent evaporated in a vacuum desiccator. Plasma (1.5 ml) was then added and vortex-mixed with ethanol (150 μl). Steroids were extracted by vortex-mixing with 3 × 2 ml

portions of *n*-pentane–ethyl acetate (1:1, v/v). The pooled extracts were washed once with 2 M NaOH (1 ml) then with water (1 ml). After transferring the washed organic phase to clean tubes, a solution in ethanol (20 μl) of Sudan IV dye contaminant was added and solvents evaporated in a rotary evaporator at 40°C with water-pump vacuum.

The dried extracts were subjected to t.l.c. as described above and the zone containing the dye contaminant (together with an-α) removed and the steroid eluted with acetone–chloroform (2:1, v/v). After evaporation to dryness, the residues were dissolved in dry benzene (1 ml) and duplicate 100 μl aliquots transferred to glass tubes. An assay was then performed in a similar manner to that described for the standard curve. A further 500 μl of the benzene solution was placed in a counting vial containing xylene-diphenyloxazole scintillant (5 ml) and radioactivity counted so that procedural losses could be corrected.

RESULTS

Antibodies. Before immunization a dilution of 1:400 of the rabbit serum (0.5 ml) bound only 5% of 5α-androstenone. After 18 weeks, however, the same vol. (0.5 ml) of a 1:4000 dilution bound 40%. The latter serum was precipitated with respect to BSA antibodies and was used in the study. After precipitation of these, the serum was stored at −20°C in 2 ml portions of 1:2 dilution.

Specificity. Cross-reactions with other steroids were estimated as described by Abraham [20] and are shown in Table 1.

Standard curves. Figure 1 shows the standard curve obtained for 5α-androstenone (10–1000 pg). As there was a high crossreaction (42%) with an-α, a standard curve for this compound was also prepared (Fig. 2) when an-α was substituted for 5α-androstenone to displace the [5α-^{3}H]-5α-androstenone (see Experimental section). The bound and free [5α-^{3}H]-5α-androstenone were separated with dextran-coated charcoal suspension and, in control experiments, only 3.7 ± 0.27% (mean ± S.D.) of the total free steroid remained.

Table 1. Percentage cross-reaction for 5α-androst-16-en-3α-ol with various steroids to an antiserum raised in rabbits to 5α-androst-16-en-3-(O-carboxymethyl) oxime-BSA conjugate. Cross-reaction was determined according to Abraham [20]

Steroid	Cross-reaction (%)
5α-Androst-16-en-3α-ol	100
4,16-Androstadien-3-one	56.7
5α-Androst-16-en-3β-ol	38.4
5,16-Androstadien-3β-ol	2.83
5α-Dihydrotestosterone	0.60
4-Androstenedione	0.38
Progesterone	0.09
Pregnenolone	0.05

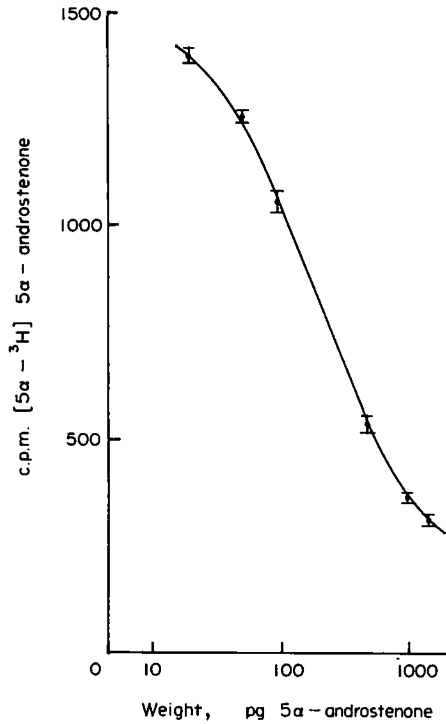

Fig. 1. Semi-logarithmic standard curve for 5α-androst-16-en-3-one. Each point represents the mean of six replicates and the bars represent ± S.D.

Accuracy. The accuracy of the method was determined by estimating the an-α content of a large volume of plasma taken from a castrated male pig. Then 500, 1000, 2500 and 5000 pg an-α were added

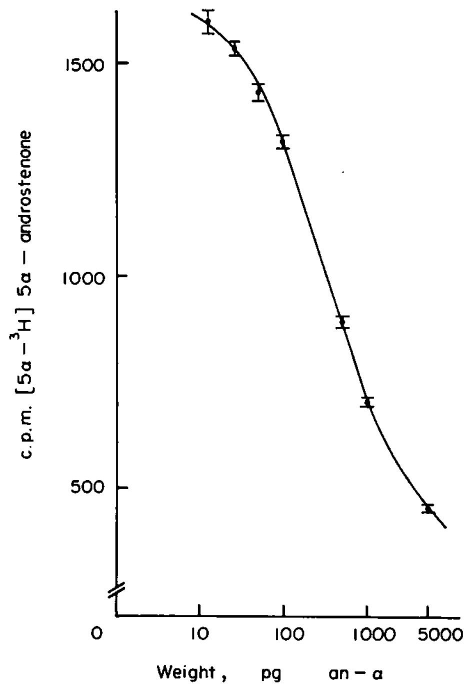

Fig. 2. Semi-logarithmic standard curve for 5α-androst-16-en-3α-ol. Each point represents the mean of six replicates and the bars represent ± S.D.

Table 2. Recovery of 5α-androst-16-en-3α-ol added to aliquots (1.5 ml) of a plasma pool taken from a castrated male pig. The basal level of 5α-androst-16-en-3α-ol was 569.8 pg/ml

5α-Androst-16-en-3α-ol added (pg)	5α-Androst-16-en-3α-ol recovered (less basal level) (pg ± S.D.)	Coefficient of variation (%)
500	581 ± 117	20.2
1000	1157 ± 203	17.6
2500	2699 ± 262	9.7
5000	5043 ± 424	8.4

Regression analysis: $y = 0.986x + 151.5$ ($r = 0.999$)

to 1.5 ml aliquots of the plasma and the an-α content re-estimated. Regression analysis of the results (Table 2) gave the equation: $y = 0.986x + 151.5$ and $r = 0.999$.

Precision. The intra-assay precision of the method was estimated by measuring the plasma an-α in two pools of peripheral blood plasma taken from castrated male pigs. Five assays were set up in duplicate on different days and the an-α values were found to be 569.8 ± 89.21 pg/ml (mean ± S.D.) and 898 ± 85.6 pg/ml (mean ± S.D.). These results gave coefficients of variation of 15.66% and 9.5% respectively. Similarly plasma samples from a man and a woman were assayed five times and found to have an-α contents of 1225.6 ± 117.6 pg/ml for the man and 506.2 ± 38.4 pg/ml for the woman (means ± S.D.). The coefficients of variation were 9.57% and 9.56% respectively.

Sensitivity. The sensitivity of the standard curve is between 10 and 20 pg. However, because of the inclusion of the separation step using t.l.c., the recovery prior to assaying is only 40%. This means that the smallest weight which can be estimated from an unknown sample is 200–300 pg, after correction for blank and recovery. Plasma samples with low an-α content (below 1 ng/ml) were re-estimated taking a larger volume of plasma.

An-α content of plasma from humans and pigs. Table 3 summarizes the results obtained.

Table 3. Mean values for the 5α-androst-16-en-3α-ol (an-α) content of plasma from adult humans and pigs

	Sex	No. of samples analysed	Mean 5α-androst-16-en-3α-ol (ng/ml)	Range
Humans	Male	31	3.08	0.3–14.9
	Female	16	0.66	0–2.4
Pigs	Male (boars)	7	30.7	10.9–58.9
	Female (gilts)	6	0.24	0.08–0.77
	Male castrates	9	0.27	0.06–0.51

DISCUSSION

The anti-serum that has been prepared in the present work for 5α-androstenone has a relatively low cross-reaction with androstadienone (23.8%) but a much higher cross-reaction with an-α (42%). This is in contrast to the antiserum developed earlier [10] which was raised to a similar 5α-androst-16-en-3-(O-carboxymethyl) oxime—BSA conjugate. Although our antiserum shows a lower specificity, employing a purification step, a useful assay has resulted. Thin-layer chromatographic purification has been used successfully in RIA's for other steroids [21–23]. Doerr [24] has indicated that, with low concentrations of steroid encountered in RIA, a thin-layer chromatographic step can produce decomposition of, and artifact formation from, the steroid to be measured. The amounts of an-α encountered in human and pig plasma (Table 3), however, are probably sufficiently high for this not to be a problem in our experiments.

Initially, it was hoped that an assay specific for 5α-androstenone could have been developed but a purification technique suitable for this steroid has not been found. Either high blanks or interference from unknown compound(s) in plasma, so far unresolved from 5α-androstenone, have prevented the development of such an assay. However, by exploiting the cross-reaction of an-α with the antiserum, an assay for this steroid has been produced. There is the possibility, however, of an incomplete separation of an-α (R_f 0.68 ± 0.02) and androstadienone (R_f 0.74 ± 0.04) and, if this were so, the plasma an-α might be elevated. Plasma androstadienone levels are likely to be very low [30], so that any interference from this source may be minimal. Both the assay and precision have been investigated and found to be acceptable. It is relevant here to refer to earlier work [25] in which an antiserum to oestradiol-17β has been utilised for measuring oestrone in addition to oestradiol-17β.

Except for the tentative identification of an-α in the peripheral plasma of a woman with adrenocortical carcinoma [26], plasma an-α has not been measured previously in men and women. The values obtained (Table 3) were found to be significantly higher in adult men than in women and this seems to correlate with the higher urinary excretion of an-α in men compared with women [4]. The plasma an-α levels are also very comparable with those of testosterone found earlier [18] *viz.*, 5.73 and 0.53 ng/ml for adult men and women respectively.

The data presented in Table 3 might suggest that an-α is related biosynthetically to testosterone but, in preliminary studies (Bicknell and Gower, unpublished observations), no correlation was found between the amounts of an-α and testosterone in the peripheral plasma of adult men. This is in keeping with studies of 16-androstenes in human testis [27] and adrenal [26] preparations when testosterone was not found to serve as a precursor for this group of steroids. The significance of these relatively large

amounts of an-α in human plasma is still unknown but experiments are in progress in this laboratory to attempt to clarify the situation.

The significance of 5α-androstenone and an-α as pheromones in pigs has been reported [2, 3] and the evidence for the testicular origin of these steroids is well-documented [15, 28]. The values for an-α obtained in intact boars (Table 3) are similar to those for 5α-androstenone [10]. The latter is reduced to an-α in boar testis [14] and both steroids are often found together in other tissues such as fat [9], salivary glands [29] and saliva [15]. In future experiments it would be of interest to study the metabolic relationship of an-α and 5α-androstenone by measuring both compounds in the peripheral plasma of the same animal. The greatly reduced values of an-α found in gilts and male castrates are also similar to those found for 5α-androstenone [10] and provide further evidence for the testicular origin of an-α in the pig.

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